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(54) Title: ADRENAL DYSFUNCTION

(57) Abstract: The invention provides methods and materials involved in the analysis of multiple components of a biochemical pathway. Specifically, the invention provides methods and materials for diagnosing adrenal dysfunction. These methods and materials involve the simultaneous analysis of multiple adrenal pathway components using high pressure liquid chromatography-tandem mass spectrometry. In addition, the invention provides methods and materials involved in identifying compounds that influence a steroid pathway in a mammal (e.g., human).

ADRENAL DYSFUNCTION

BACKGROUND

1. Technical Field

The invention relates to methods and materials involved in the diagnosis of adrenal dysfunction. Specifically, the invention involves diagnosing adrenal dysfunction by simultaneously analyzing multiple adrenal pathway components using high pressure liquid chromatography-tandem mass spectrometry (LC/MS/MS).

2. Background

The adrenals are two crescent-shaped glands positioned over the upper pole of each kidney. Each adrenal gland consists of internal layers that produce different substances. The inner part, or adrenal medulla, secretes epinephrine and norepinephrine, more commonly known as adrenaline and noradrenaline. These hormones are the "fight or flight" hormones that are released in potentially life or death situations. Their release increases heart rate and blood pressure, and diverts more blood to the brain, heart, and skeletal muscles. The adrenal cortex lies outside the adrenal medulla and responds to a different type of stress. Steroid hormones such as cortisone, testosterone, estrogen, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, DHEA, pregnenolone, aldosterone, androstenedione, and progesterone are produced in the adrenal cortex by enzymes that convert steroid substrates into products along a steroid synthesis pathway. Deficiencies in the conversion enzymes along a steroid synthesis pathway can lead to adrenal dysfunction, including 11 β-hydroxylase deficiency, 21-hydroxylase deficiency, Addison's disease, and Cushing's disease.

SUMMARY

The invention involves the use of LC/MS/MS to diagnosis adrenal dysfunction by simultaneously analyzing multiple components in the adrenal pathway. This technology can make the differential diagnosis of enzyme deficiencies in congenital adrenal hyperplasia (CAH) much simpler than conducting various immunoassays. Tandem mass spectrometry gives positive identification to each metabolic component and is superior to

immunoassays, which lack the positive identification. In addition, immunoassays can have cross-reactivity interference caused by structurally related compounds in the pathway or with drugs of similar structure. For example, a very small percentage of cross-reactivity can lead to an incorrect diagnosis. The data presented herein demonstrate that LC/MS/MS can be used to diagnose adrenal dysfunction accurately when multiple adrenal pathway components are simultaneously analyzed. Further, using multiple determinations from a single sample avoids the problem of having excessive amounts of blood being drawn from the patient.

In general, the invention features a method for determining whether a mammal (e.g., human or rodent) has adrenal dysfunction. The method involves simultaneously determining the levels of at least three adrenal pathway components in a sample from the mammal using LC/MS/MS, where an elevation or reduction in at least one of the levels compared to normal levels indicates adrenal dysfunction. The adrenal dysfunction can include decreased or increased enzyme activity. The adrenal dysfunction can be an 11 β-hydroxylase deficiency, a 21-hydroxylase deficiency, Addison's disease, Cushing's disease, a 17 α-hydroxylase deficiency, or a 3 β-hydroxylase deficiency. The pathway components can be pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone, androstene-Δ^4 dione, androstene-Δ^5 diol, or 21 deoxycortisol. The at least three adrenal pathway components can be any three of the following: pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, dehydroepiandrosterone, androstene-Δ^4 dione, androstene-Δ^5 diol, and 21 deoxycortisol. The at least three adrenal pathway components can be any three of the following: pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, dehydroepiandrosterone, androstene-Δ^4 dione, and 21 deoxycortisol. The at least three adrenal pathway components can be any three of the following: 11-deoxycorticosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, and 21 deoxycortisol. The at least three adrenal pathway components can be any three of the following: 11-deoxycorticosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, and 21 deoxycortisol.

The method can involve simultaneously determining the levels of at least four (or at least five, six, seven, eight, nine, or more) adrenal pathway components in the sample using LC/MS/MS.

In another embodiment, the invention features a method of assisting a person (e.g., medical or research professional) in determining the presence of adrenal dysfunction in a mammal (e.g., human or rodent). The method involves (a) simultaneously determining the levels of at least three adrenal pathway components in a sample from the mammal using LC/MS/MS, and (b) communicating information about the levels to the person, wherein an elevation or reduction in at least one of the levels compared to normal levels indicates adrenal dysfunction. The adrenal dysfunction can be an 11 β-hydroxylase deficiency, a 21-hydroxylase deficiency, Addison's disease, Cushing's disease, a 17 α-hydroxylase deficiency, or a 3 β-hydroxylase deficiency. The adrenal pathway components can be pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone, androstene-Δ^4 dione, androstene-Δ^5 diol, or 21 deoxycortisol. The sample can be a blood or a serum sample. The communication can include sending the information directly or indirectly to the person. The communication can include making the information electronically available to the person.

In another aspect, the invention features a method for identifying a compound that affects a steroid pathway in a mammal. The method includes (a) obtaining a sample from the mammal after the mammal was treated with the compound, and (b) simultaneously determining the levels of at least three adrenal pathway components in the sample using LC/MS/MS, where an elevation or reduction in at least one of the levels compared to normal levels indicates that the compound affects the steroid pathway (e.g., adrenal pathway or gonadal pathway). The compound can be an animal-derived compound, a plant-derived compound, or an herbal medicine.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents,

and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is flow chart depicting the adrenal pathway as well as an entry point to the gonadal pathway. The dotted lines (. . . .) indicate enzyme action.

Figure 2 is a bar graph plotting the amount of the indicated adrenal pathway components detected in a sample from a patient with normal response during (1-24) ACTH stimulation. Specimens were collected at basal level (before injection of ACTH) as well as 30 minutes and 60 minutes after ACTH injection. The dose of (1-24) ACTH (cosyntropin) was 0.5 mg/patient.

Figure 3 is a bar graph plotting the amount of the indicated adrenal pathway components detected in a sample from a patient with normal response during (1-24) ACTH stimulation. Specimens were collected at basal level (before injection of ACTH) as well as 30 minutes and 60 minutes after ACTH injection. The dose of (1-24) ACTH (cosyntropin) was 0.5 mg/patient.

Figure 4 is a bar graph plotting the amount of the indicated adrenal pathway components detected in a sample from an Addison's disease patient after ACTH stimulation. Essentially, there was no change in steroid concentration during stimulation. The dose of (1-24) ACTH (cosyntropin) was 0.5 mg/patient.

Figure 5 is a bar graph plotting the amount of the indicated adrenal pathway components detected in a sample from a patient before and after receiving metyrapone. Three grams of Metyrapone was taken at 11 p.m. the night before the second sample was drawn the next morning.

Figure 6 is a bar graph plotting the amount of the indicated adrenal pathway components detected in a sample from a patient before and after receiving metyrapone. Three grams of Metyrapone was taken at 11 p.m. the night before the second sample was drawn the next morning.

Figure 7 is a bar graph plotting the amount of the indicated adrenal pathway components detected in a sample from a subject before, on day 1, and day 7 after ingesting 1000 mL hot water (4 x 250 mL) wherein a 2 g licorice root cutting was steeped. The water was ingested over 8 hours, and the process was repeated every day with a fresh licorice root cutting for 7 days.

DETAILED DESCRIPTION

The invention provides methods and materials related to the use of LC/MS/MS to diagnose adrenal dysfunction by simultaneously analyzing multiple components in the adrenal pathway. Adrenal dysfunctions include, without limitation, 11 β-hydroxylase deficiencies, 21-hydroxylase deficiencies, Addison's disease, Cushing's disease, 17 α-hydroxylase deficiencies, and 3 β-hydroxylase deficiencies. An adrenal dysfunction can be diagnosed from either elevated or reduced levels of an adrenal pathway component. It is appreciated that adrenal pathway components are pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone, androstene-Δ^4 dione, androstene-Δ^5 diol, and 21 deoxycortisol. An adrenal dysfunction also can be diagnosed from either elevated or reduced levels of an adrenal pathway-related component. Adrenal pathway-related components include any non-adrenal pathway component that is useful for assessing adrenal function.

The invention provides methods for determining the presence of adrenal dysfunction by simultaneously analyzing multiple adrenal pathway or adrenal pathway-related components. To determine the presence of adrenal dysfunction, the levels of at least three (e.g., three, four, five, six, seven, eight, nine, or ten) adrenal pathway components or adrenal pathway-related components or combinations thereof in a sample from a patient can be measured simultaneously using LC/MS/MS. For example, LC/MS/MS can be used to determine the presence of a 17 α-hydroxylase deficiency by measuring an elevated level of 11 deoxycorticosterone (DOC) without observing an elevation in 11-deoxycortisol levels. In another example, LC/MS/MS can be used to determine the presence of an 11 β-hydroxylase deficiency by measuring an elevated level of both DOC and 11-deoxycortisol. Patients with 11 β-hydroxylase deficiency or 17 α-

hydroxylase deficiency have reduced levels of cortisol and cortisone. In a further example, LC/MS/MS can be used to determine the presence of a 21-hydroxylase deficiency by measuring an elevated level of both 17α-hydroxyprogestrone and 21-deoxycortisol.

An elevated level of an adrenal pathway or adrenal pathway-related component refers to any level that is greater than a reference level for that particular adrenal pathway or adrenal pathway-related component. In addition, a reduced level of an adrenal pathway or adrenal pathway-related component refers to any level that is less than a reference level for that particular adrenal pathway or adrenal pathway-related component. It is appreciated that a reference level of an adrenal pathway or adrenal pathway-related component refers to the level of that particular component typically measured in samples from mammals lacking adrenal dysfunction. For example, a reference level of 17-hydroxyprogesterone can be the average level of 17-hydroxyprogesterone that is present in samples obtained from a random sampling of 50 subjects lacking adrenal dysfunction. Reference levels for adrenal pathway components such as 11-deoxycorticosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, 21 deoxycortisol, cortisol, and cortisone can be determined as set forth in Example 1.

It is appreciated that levels from comparable samples are used when determining whether or not a particular level is an elevated or a reduced level. For example, the average level of 11 β-deoxycortisol present in serum samples from a random sampling of subjects lacking 11 β-hydroxylase deficiency may be 1 μg/dL of serum, while the average level of 11 β-deoxycortisol present in whole blood samples from the same random sampling of subjects may be 0.5 μg/dL of whole blood. In this case, the reference level for 11 β-deoxycortisol in serum would be 1 μg/dL of serum, and the reference level for 11 β-deoxycortisol in whole blood would be 0.5 μg/dL of whole blood. Thus, when determining whether or not the level of 11 β-deoxycortisol measured in serum is elevated, the measured level would be compared to the reference level for 11 β-deoxycortisol in serum (i.e., 1 μg/dL of serum). Additionally, a reference level can be any amount. For example, a reference level for 11 β-deoxycortisol in serum can be zero. In this case, any level of 11 β-deoxycortisol in serum greater than zero would be an elevated level.

Any sample can be used to measure multiple adrenal pathway or adrenal pathway-related components. For example, blood, serum, or plasma can be used to measure such components. In addition, any method can be used to obtain a sample. For example, a whole blood sample can be obtained by venipuncture. Once obtained, a sample can be manipulated prior to measuring the levels of adrenal pathway or adrenal pathway-related components. For example, a whole blood sample can be centrifuged such that serum is obtained. Once obtained, the serum can be extracted before LC/MS/MS analysis.

Another enzyme deficiency that is involved in adrenal dysfunction is the lack of 3 β-hydroxylase/$\Delta^{4,5}$ isomerase activity. Two types of this enzyme exist. Type 1 is present at peripheral organs, skin, breast, and placenta, whereas type 2 is in the adrenal gland, testes, and ovaries. When the adrenal gland deficiency is of type 2, the peripheral enzyme (type 1) can assume some of the function. Two defective alleles are required to manifest the biochemical and clinical deficiency of 3 β-hydroxylase making the phenotype deficiency rare.

In one embodiment, LC/MS/MS can be performed on an extracted serum sample. Pregnenolone (mother: m/z 317.0 and daughter: m/z 299.1), progesterone (mother: m/z 315.2 and daughter: m/z 109.1), 11-deoxycorticosterone (mother: m/z 331.2 and daughter: m/z 109.1), corticosterone (mother: m/z 347.3 and daughter: m/z 328.8), aldosterone (mother: m/z 360.44), 17α-hydroxypregnenolone (mother: m/z 331.1 and daughter: m/z 297.4), 17α-hydroxyprogesterone (mother: m/z 331.2 and daughter: m/z 109.1), 11-deoxycortisol (mother: m/z 347.2 and daughter: m/z 109.1), cortisol (mother: m/z 363.0 and daughter: m/z 121.1), cortisone (mother: m/z 361.0 and daughter: m/z 163.0), dehydroepiandrosterone (mother: m/z 330.47), androstene-Δ^4 dione (mother: m/z 287.4 and daughter: m/z 97.2), androstene-Δ^5 diol (mother: m/z 290.43), and 21-deoxycortisol (mother: m/z 347.1 and daughter: m/z 311.2) can be detected in conjunction with d_4-cortisol (mother: m/z 366.9 and daughter: m/z 121.2) as an internal standard. The analysis can include a calibration curve extending over a desired detection range. When using this method to analyze serum samples from normal subjects, only cortisol and cortisone can be quantitatively determined. In serum samples from patients with known enzyme deficiencies, however, elevated levels of adrenal pathway components can be measured within the analytical detection limits of the instrumentation. The LC/MS/MS

methods provided herein correlate with an established LC/UV procedure for detecting cortisol.

In another embodiment, tests of 24-hour urinary free cortisol can be used to measure the output of cortisol secretion to diagnose Cushing's syndrome. Briefly, the use of HPLC for the determination can eliminate interference from drugs and other compounds that interfere with immunoassays so that one can differentiate Cushing's syndrome from healthy subjects. Using HPLC analysis, both cortisol and cortisone can be measured and reported simultaneously. With the one parameter of cortisol, the efficacy of the test to detect Cushing's syndrome is 76 percent. With one parameter of cortisone, the efficacy is 85 percent. Using both parameters, the efficacy increases to 90 percent. The use of HPLC not only eliminates interference, but also increases test efficacy.

The methods and materials described herein can be used to identify compounds that influence a steroid pathway. The term "steroid pathway" as used herein refers to any steroid synthesis or degradation pathway. For example, the adrenal and gonadal pathways are steroid pathways. Any compound can be analyzed using these methods and materials. For example, drugs, food products, animal-derived compounds, plant-derived compounds, and herbal medicines can be analyzed for the ability to influence a steroid pathway. In addition, the methods and materials described herein can be used to evaluate compounds for any potential side-effects caused by influencing the steroid pathway, thus speeding up clinical trials. For example, synthetic drugs can be rapidly evaluated to determine whether they influence the P450 isoenzymes of the adrenal pathway. Likewise, herbal medicines such as licorice can be tested for any influences they may have on a steroid pathway.

The invention also provides methods and materials to assist medical or research professionals in determining the presence of adrenal dysfunction in a mammal. Medical professionals can be, for example, doctors, nurses, medical laboratory technologists, and pharmacists. Research professionals can be, for example, principle investigators, research technicians, postdoctoral trainees, and graduate students. A professional can be assisted by (1) simultaneously determining the levels of at least three adrenal pathway

components in a sample using LC/MS/MS, and (2) communicating information about one or more of those levels to that professional.

Any method can be used to communicate information to another person (e.g., a professional). For example, information can be given directly or indirectly to a professional. In addition, any type of communication can be used to communicate the information. For example, mail, e-mail, telephone, and face-to-face interactions can be used. The information also can be communicated to a professional by making that information electronically available to the professional. For example, the information can be communicated to a professional by placing the information on a computer database such that the professional can access the information. In addition, the information can be communicated to a hospital, clinic, or research facility serving as an agent for the professional.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – Diagnosis of adrenal dysfunction

A 500 μL serum sample from a patient was spiked with 190 pmole of d_4-cortisol (Cambridge Isotopes Laboratories, Inc.) as an internal standard. The sample was then extracted with methylene chloride. The extracted sample was used for LC/MS/MS analysis. Briefly, the extracted sample was separated by chromatography on a reverse-phase column (Supelcosil LC-18, 33 x 4.6 mm) using 50/45 methanol/water at 1.0 mL/min. The resulting separated sample proceeded in-line directly to a tandem mass spectrometer (API 2000; Perkin-Elmer Sciex) operating in the multiple reaction monitoring mode. Pregnenolone (m/z 317.0 and m/z 299.1), progesterone (m/z 315.2 and m/z 109.1), 11-deoxycorticosterone (m/z 331.2 and m/z 109.1), corticosterone (m/z 347.3 and m/z 328.8), 17α-hydroxypregnenolone (m/z 331.1 and m/z 297.4), 17α-hydroxyprogesterone (m/z 331.2 and m/z 109.1), 11-deoxycortisol (m/z 347.2 and m/z 109.1), cortisol (m/z 363.0 and m/z 121.1), cortisone (m/z 361.0 and m/z 163.0), androstene-Δ^4 dione (m/z 287.4 and m/z 97.2), and 21-deoxycortisol (m/z 347.1 and m/z 311.2) were detected in conjunction with d_4-cortisol (m/z 366.9 and m/z 121.2) as an

internal standard. All results were generated in the positive ion mode. The analysis time was seven minutes, and three separate ion-monitoring windows was utilized to maximize sensitivity (Weinmann *et al.*, *J. Am. Soc. Mass Spectrom.* 10:1028-1037 (1999)). The analysis included a 9-point calibration curve extending from 0.5-25 μg/dL. The lower level of detection was about 0.5 μg/dL. A comparison of methods for cortisol against an established LC/UV procedure (X) resulted in Y=1.0647 X + 0.1343, r = 0.9940, N = 27. In serum samples from normal subjects, only cortisol and cortisone could be quantitatively determined. In serum samples from patients with known enzyme deficiencies, however, elevated levels of adrenal pathway components were measured within the analytical detection limits of the instrumentation.

The clinical value of this method was demonstrated by analyzing serum samples of confirmed patients with 11 β-hydroxylase deficiency (n=3), 21-hydroxylase deficiency (n=2), and patients receiving either metyrapone inhibition (n=2) or ACTH stimulation (n=3) in the context of diagnostic procedures.

The two healthy subjects both exhibited cortisol and cortisone in their plasma. One subject had only a trace amount (0.3 μg/dL) of 11-deoxycortisol. In plasma, the amount of cortisol is greater than its inactive metabolite cortisone (Table 1).

The three patients with 11 β-hydroxylase deficiency exhibited trace amounts of cortisol and cortisone, but had high levels of 11 β-deoxycortisol, which is the hallmark of 11 β-hydroxylase deficiency. A noticeable amount of 11-deoxycorticosterone (DOC) was present, which was also caused by 11 β-hydroxylase deficiency (Table 1). One patient also exhibited an increase in corticosterone due to the 11 β-hydroxylase deficiency (Table 1).

The two patients with 21-hydroxylase deficiency exhibited a high level of 17-hydroxyprogesterone and trace amounts of cortisol and cortisone. A measurable amount of 21-deoxycortisol was present, which is a marker of 21-hydroxylase deficiency and is not a precursor in the pathway of adrenocortical hormone biosynthesis (Figure 1). Trace amounts of 11-deoxycortisol and DOC were also present (Table 1). One patient also exhibited an increase in 17-hydroxypregnenolone due to the 21-hydroxylase deficiency (Table 1).

Two patients with normal response during (1-24) ACTH stimulation exhibited an increase of cortisol. Cortisol peak occurred either at 30 or 60 minutes after receiving (1-24) ACTH injection. Little change was seen for the precursors (Figures 2 and 3).

One patient with Addison's disease, whose ACTH was 1900 pg/mL (reference range <23 pg/mL) exhibited no ACTH stimulation effect on cortisol or precursors at either 30 or 60 minutes (Figure 4).

Two patients underwent overnight metyrapone testing to block 11 β-hydroxylase activity. The next-morning samples exhibited highly elevated levels of 11-deoxycortisol and greatly decreased levels of cortisol (Figures 5 and 6).

The corticortropin (1-24) ACTH stimulation test revealed that the reaction was very rapid. When specimens were collected 30 and 60 minutes after stimulation, cortisol levels were increased in normal subjects. During these intervals, no intermediate compound such as 11-deoxycortisol or 17-hydroxyprogesterone was detected after ACTH stimulation. This phenomenon indicated that the precursors were converted to cortisol rapidly. The results for the pharmaceutical block of 11 β-hydroxylase by metyrapone revealed a significant increase in intermediate compounds 11-deoxycortisol and DOC and a decreased in the end product cortisol. Taken together, the data presented herein indicate that measuring only intermediate-compounds or precursors was clinically insignificant. In addition, the data indicated that LC/MS/MS had the ability to diagnose enzyme deficiency, even with a sensitivity of 0.1 μg/dL. ACTH stimulates P450scc isoenzyme, which converts cholesterol (C_{21}) to pregnenolone (C_{19}) starting the process of the adrenal pathway. Metyrapone blocks P450c 11 isoenzyme activity by converting 11-doxycortisol to cortisol. The experiments using these compounds demonstrate that the methods and materials described herein can be used to identify compounds that either stimulate certain reactions or inhibit certain reactions in a steroid pathway.

Table 1. Adrenal pathway components measured by LC/MS/MS

	PL	PG	DOC	CS	17-HPL	17-HPG	11-DC	Cortisol	Cortisone	AD	21-DC
Normals*			nd			nd	nd	14.3 ± 8.1			nd
†Subject #1			nd			nd	nd	13.6	2.1		nd
†Subject #2			nd			nd	0.3	8.9	0.6		nd
‡Patient #1			3.9			0.9	14.9	0.5	nd		nd
‡Patient #2			1.0			1.2	10.0	0.5	nd		nd
§Patient #3	0.02	0.11	0.28	1.34	0.67	0.13	5.48	4.87	0.76	0.21	nd
§Patient #1			0.3			28.5	0.9	0.9	0.3		6.9
§Patient #2	0.1	0.11	nd	0.13	1.97	7.51	0.02	0.83	0.17	0.19	0.69

All metabolites were measured in serum samples. All values are in µg/dL. PL, pregnenolone; PG, progesterone; DOC, deoxycorticosterone; CS, corticosterone; 17-HPL, 17-hydroxypregnenolone; 17-HPG, 17-hydroxyprogesterone; 11-DC, 11-deoxycortisol; AD, androstene-Δ^4 dione; 21-DC, 21-deoxycortisol.

*Assays of sera from 36 healthy subjects (fasting morning specimens) gave the following results (mean ± SD).

†Normal human subject

‡Human patient with 11 β-hydroxlase deficiency

§Human patient with 21-hydroxylase deficiency

Example 2 – Identifying compounds that affect the adrenal pathway using LC/MS/MS

A 0.5 mL serum sample was taken from a healthy subject as a control. The subject was then given a 2 g licorice root cutting to steep in a 250 mL aliquot of hot water. After drinking the aliquot while retaining the licorice root, the licorice root was again steeped in another 250 mL aliquot of hot water. The subject drank a total of 4 aliquots (i.e., 1000 mL) in the course of eight hours. The subject repeated this process for seven days, using a fresh 2 g licorice root cutting every day. Serum samples (0.5 mL) from the subject were taken after day 1 and day 7. LC/MS/MS analysis was performed as described in Example 1.

Pregnenolone (m/z 317.0 and m/z 299.1), progesterone (m/z 315.2 and m/z 109.1), 11-deoxycorticosterone (m/z 331.2 and m/z 109.1), corticosterone (m/z 347.3 and m/z 328.8), 17α-hydroxypregnenolone (m/z 331.1 and m/z 297.4), 17α-hydroxyprogesterone (m/z 331.2 and m/z 109.1), 11-deoxycortisol (m/z 347.2 and m/z 109.1), cortisol (m/z 363.0 and m/z 121.1), cortisone (m/z 361.0 and m/z 163.0), androstene-Δ^4 dione (m/z 287.4 and m/z 97.2), and 21-deoxycortisol (m/z 347.1 and m/z 311.2) were detected in conjunction with d_4-cortisol (m/z 366.9 and m/z 121.2) as an internal standard.

The subject exhibited an elevated level of cortisol on day 1 (9.3 μg/dL) compared to the control level (8.51 μg/dL). In addition, an elevated level of cortisol was detected on day 7 (13.83 μg/dL) compared to the control level. The levels of ten other adrenal pathway components (pregnenolone, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, androstene-Δ^4 dione, 11-deoxycortisol, 11-deoxycorticosterone, 21-deoxycortisol, corticosterone, and cortisone) remained low in both the day 1 and day 7 samples (Figure 7).

These results indicate that licorice root inhibits the enzyme 11 β-hydroxysteroid dehydrogenase. 11 β-hydroxysteroid dehydrogenase converts cortisol to cortisone leading to an elevated level of cortisol in serum. These results demonstrate not only that LC/MS/MS can be used to detect multiple adrenal pathway components with reproducible sensitivity, but also demonstrate that LC/MS/MS can be used to discover

compounds (e.g., herbal medicines such as licorice root, drugs, plant- or animal-derived compounds, or edible products) that affect the adrenal pathway.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for determining whether a mammal has adrenal dysfunction, said method comprising simultaneously determining the levels of at least three adrenal pathway components in a sample from said mammal using LC/MS/MS, wherein an elevation or reduction in at least one of said levels compared to normal levels indicates said adrenal dysfunction.

2. The method of claim 1, wherein said adrenal dysfunction comprises decreased enzyme activity.

3. The method of claim 1, wherein said adrenal dysfunction comprises increased enzyme activity.

4. The method of claim 1, wherein said adrenal dysfunction is selected from the group consisting of 11 β-hydroxylase deficiency, 21-hydroxylase deficiency, Addison's disease, Cushing's disease, 17 α-hydroxylase deficiency, and 3 β-hydroxylase deficiency.

5. The method of claim 1, wherein said mammal is a human.

6. The method of claim 1, wherein said pathway components are selected from the group consisting of pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone, androstene-Δ^4 dione, androstene-Δ^5 diol, and 21 deoxycortisol.

7. The method of claim 1, wherein said at least three adrenal pathway components are selected from the group consisting of pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, dehydroepiandrosterone, androstene-Δ^4 dione, androstene-Δ^5 diol, and 21 deoxycortisol.

8. The method of claim 1, wherein said at least three adrenal pathway components are selected from the group consisting of pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, dehydroepiandrosterone, androstene-Δ^4 dione, and 21 deoxycortisol.

9. The method of claim 1, wherein said at least three adrenal pathway components are selected from the group consisting of 11-deoxycorticosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, and 21 deoxycortisol.

10. The method of claim 1, wherein said at least three adrenal pathway components are selected from the group consisting of 11-deoxycorticosterone, 17α-hydroxyprogesterone, 11-deoxycortisol, and 21 deoxycortisol.

11. The method of claim 1, wherein said method comprises simultaneously determining the levels of at least four adrenal pathway components in said sample using LC/MS/MS.

12. The method of claim 1, wherein said method comprises simultaneously determining the levels of at least five adrenal pathway components in said sample using LC/MS/MS.

13. The method of claim 1, wherein said method comprises simultaneously determining the levels of at least eight adrenal pathway components in said sample using LC/MS/MS.

14. A method of assisting a person in determining the presence of adrenal dysfunction in a mammal, wherein said method comprises:

a) simultaneously determining the levels of at least three adrenal pathway components in a sample from said mammal using LC/MS/MS, and

b) communicating information about said levels to said person, wherein an elevation or reduction in at least one of said levels compared to normal levels indicates said adrenal dysfunction.

15. The method of claim 14, wherein said person is a medical or research professional.

16. The method of claim 14, wherein said adrenal dysfunction is selected from the group consisting of 11 β-hydroxylase deficiency, 21-hydroxylase deficiency, Addison's disease, Cushing's disease, 17 α-hydroxylase deficiency, and 3 β-hydroxylase deficiency.

17. The method of claim 14, wherein said mammal is a human.

18. The method of claim 14, wherein said mammal is a rodent.

19. The method of claim 14, wherein said adrenal pathway components are selected from the group consisting of pregnenolone, progesterone, 11-deoxycorticosterone, corticosterone, aldosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone, androstene-Δ^4 dione, androstene-Δ^5 diol, and 21 deoxycortisol.

20. The method of claim 14, wherein said sample is a blood sample.

21. The method of claim 14, wherein said sample is a serum sample.

22. The method of claim 14, wherein said communication comprises sending said information directly to said person.

23. The method of claim 14, wherein said communication comprises sending said information indirectly to said person.

24. The method of claim 14, wherein said communication comprises making said information electronically available to said person.

25. A method for identifying a compound that affects a steroid pathway in a mammal, said method comprising:

(a) obtaining a sample from said mammal after said mammal was treated with said compound, and

(b) simultaneously determining the levels of at least three adrenal pathway components in said sample using LC/MS/MS, wherein an elevation or reduction in at least one of said levels compared to normal levels indicates that said compound affects said steroid pathway.

26. The method of claim 25, wherein said steroid pathway is an adrenal pathway.

27. The method of claim 25, wherein said steroid pathway is a gonadal pathway.

28. The method of claim 25, wherein said compound is an animal-derived compound.

29. The method of claim 25, wherein said compound is a plant-derived compound.

30. The method of claim 25, wherein said compound is an herbal medicine.

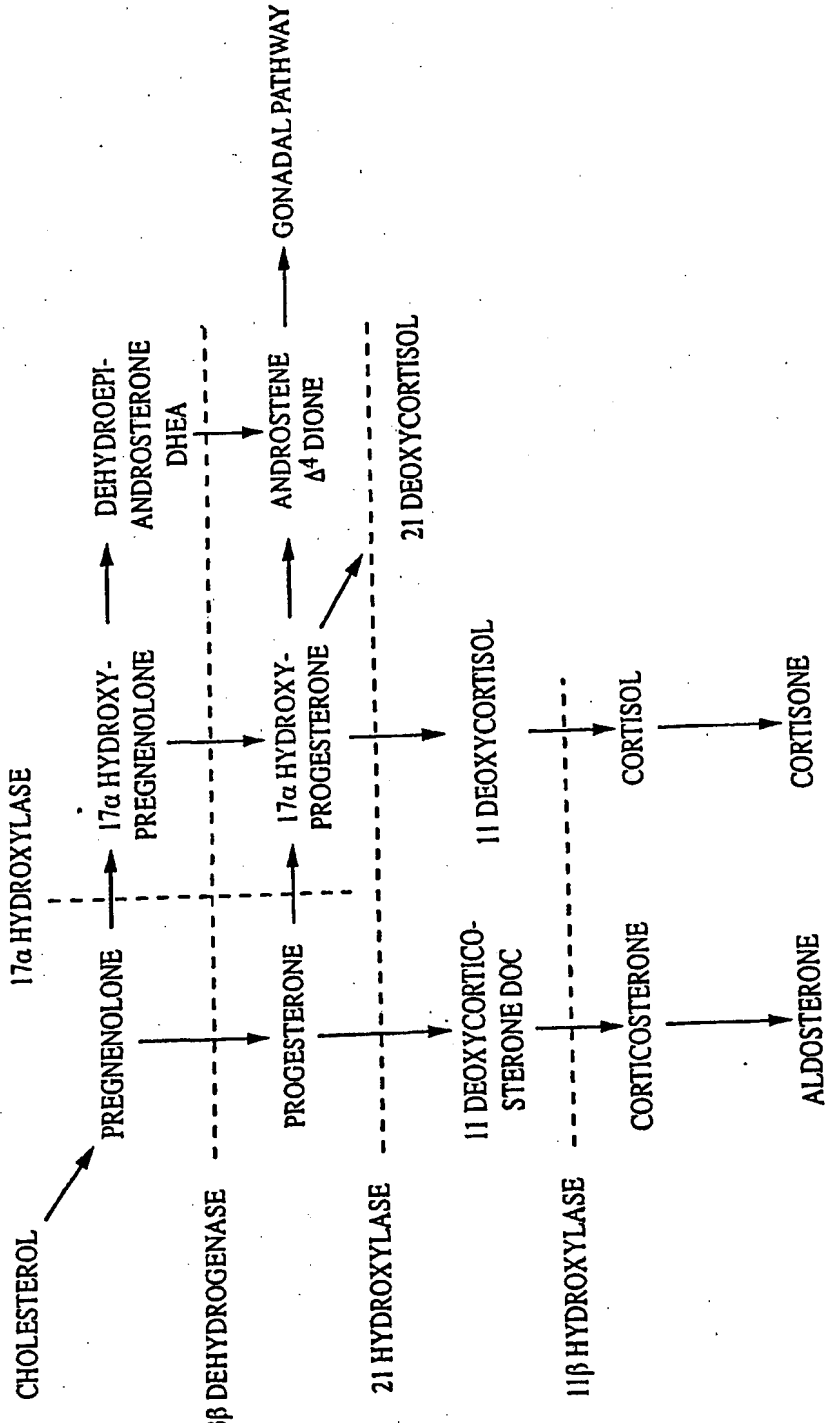

FIG. 1

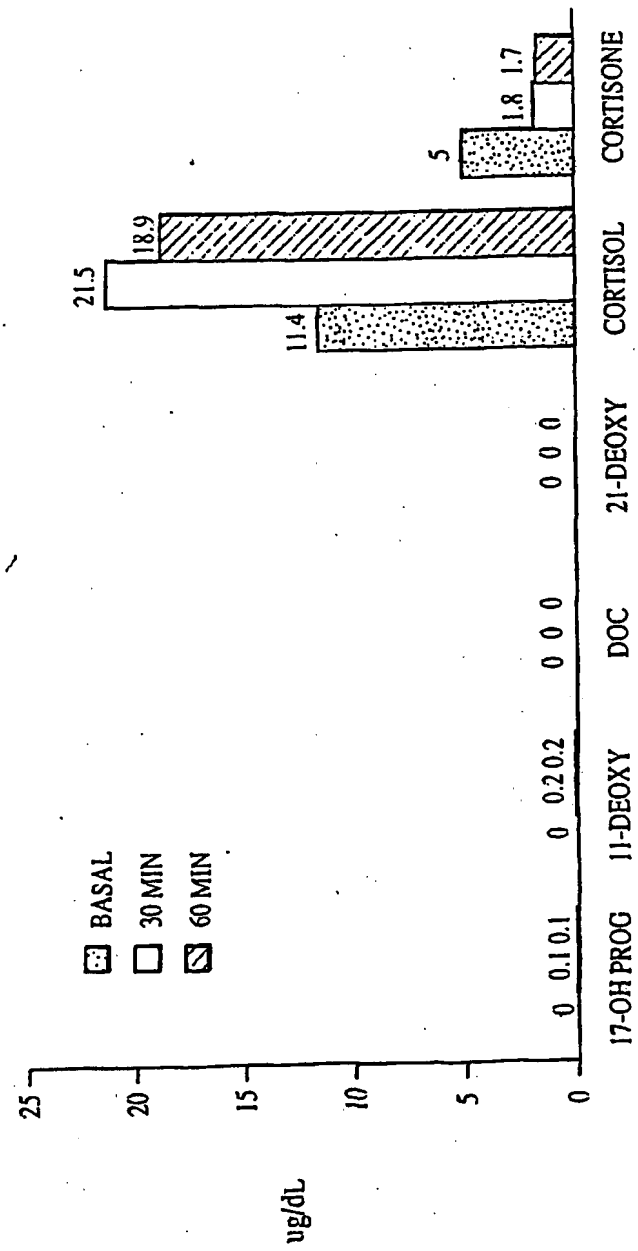

FIG. 2

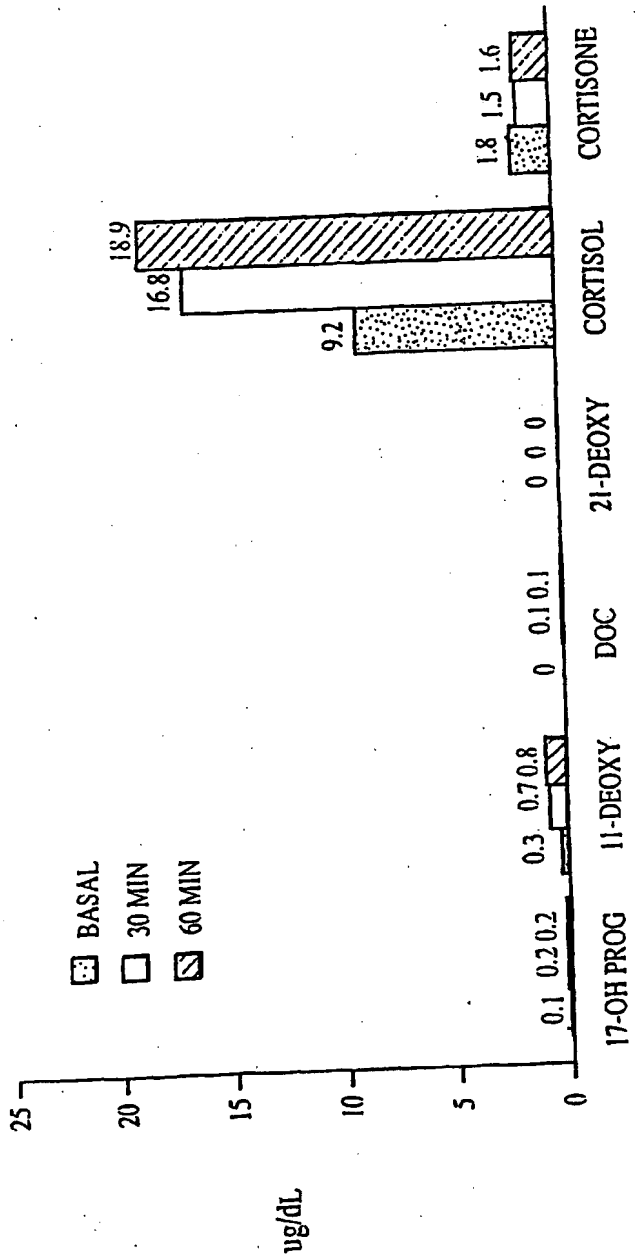

FIG. 3

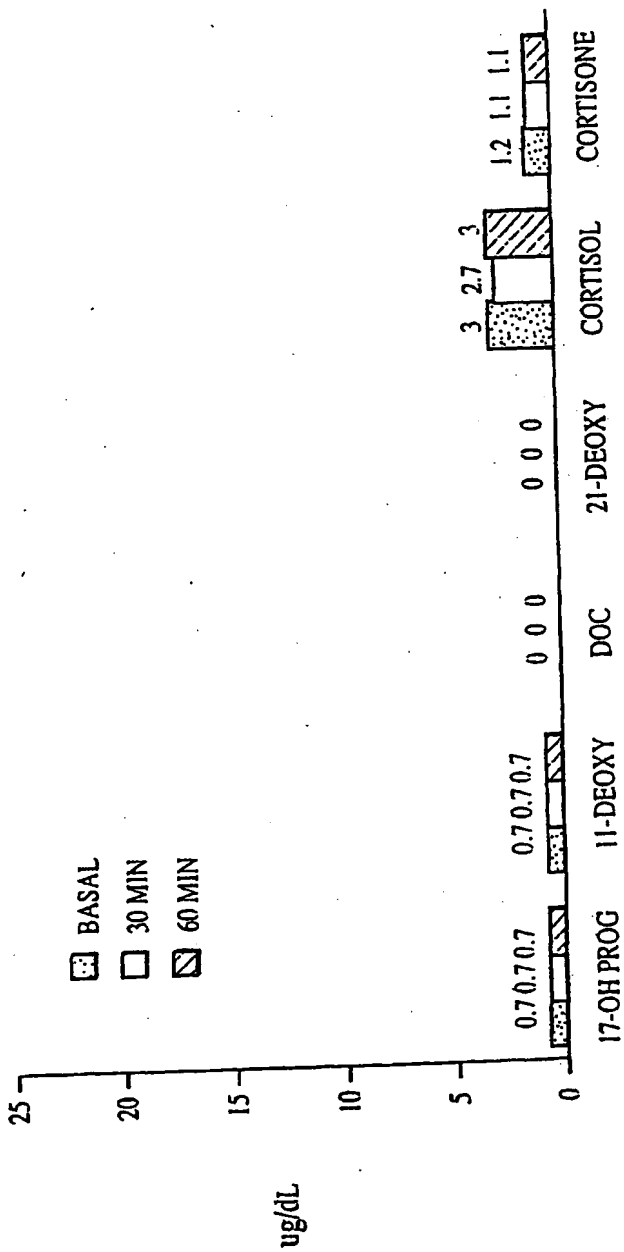

FIG. 4

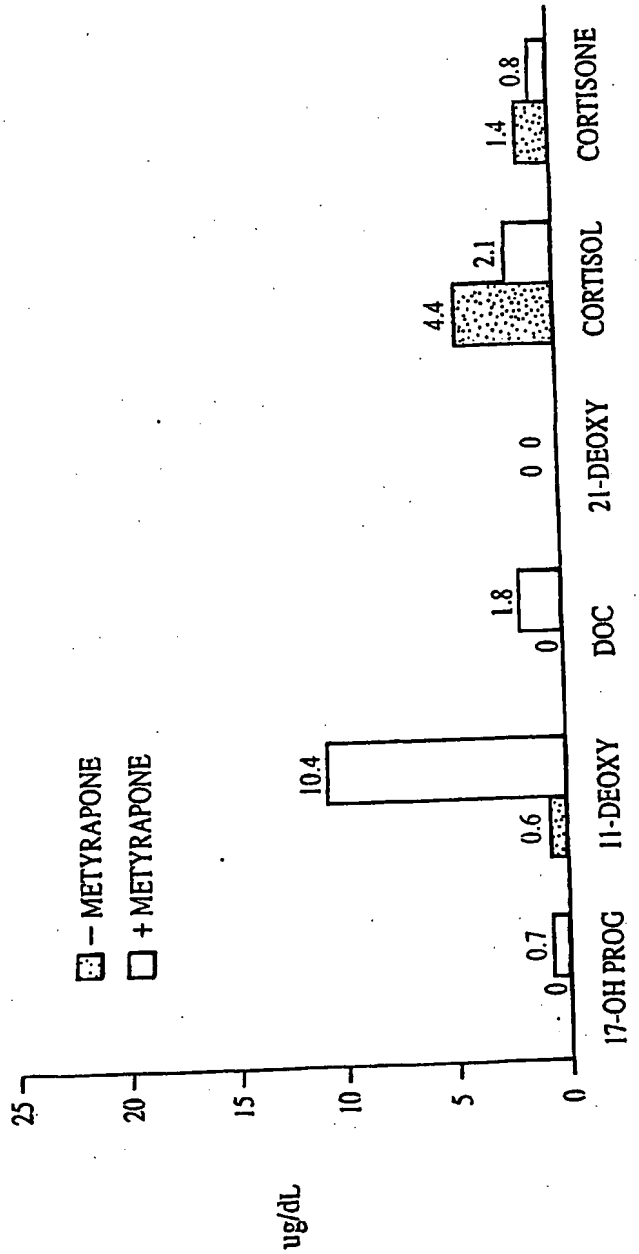

FIG. 5

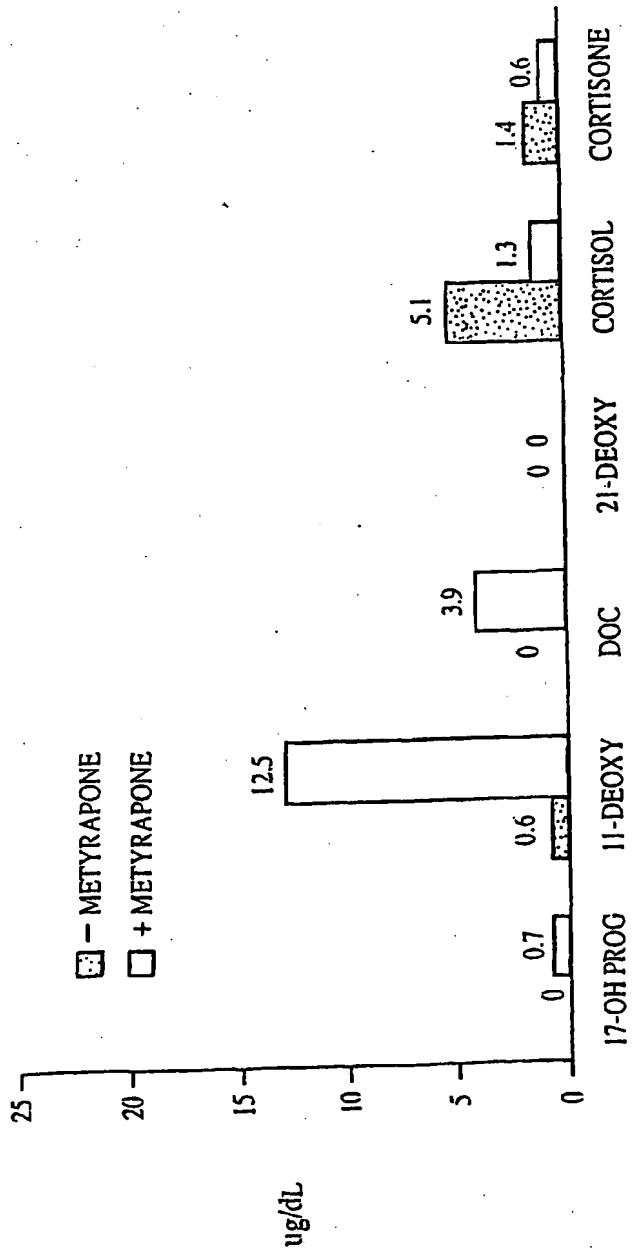

FIG. 6

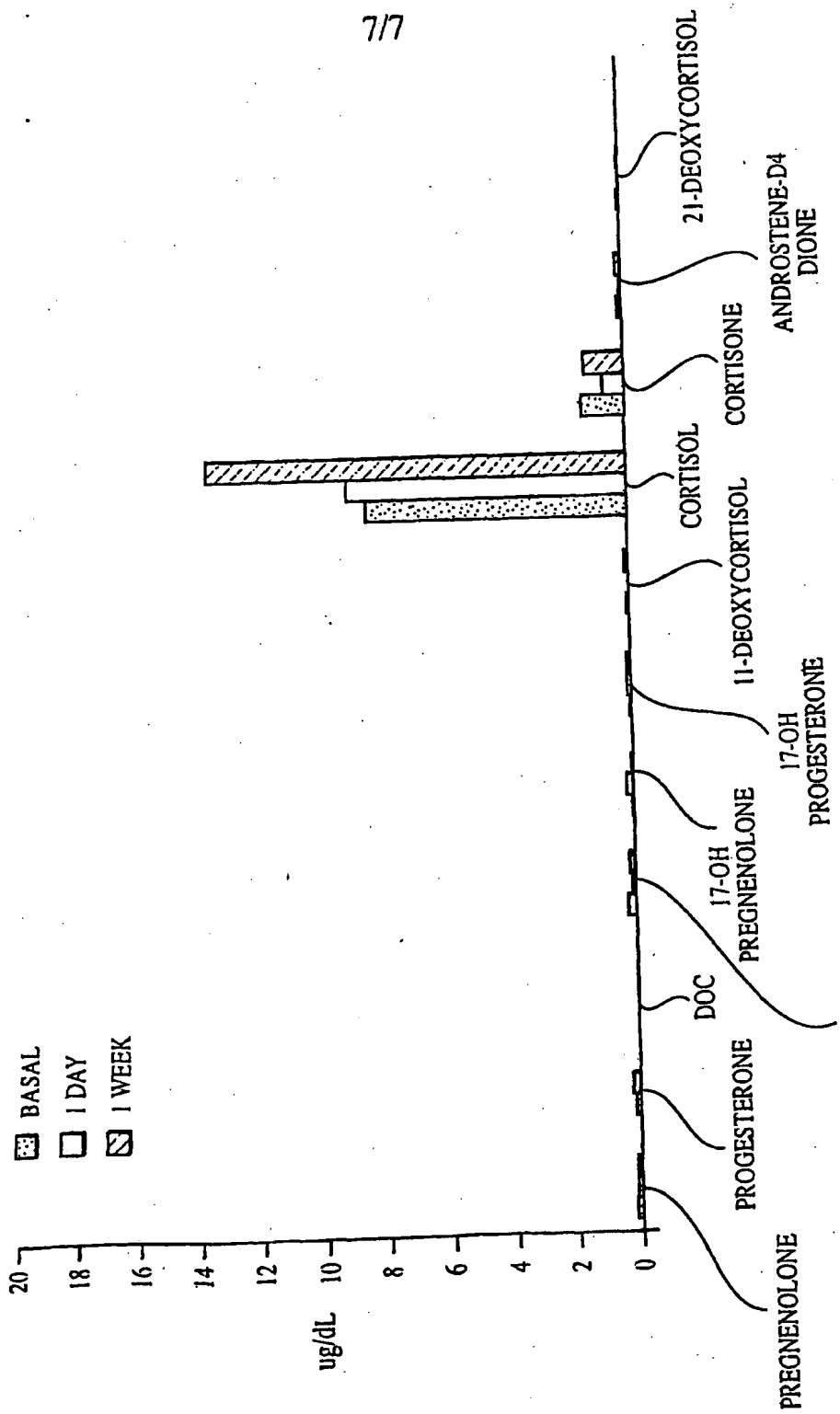

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/16028

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/16028

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-13, drawn to a method of determining if a mammal has adrenal dysfunction.

Group II, claim(s) 14-24, drawn to a method of assisting a person in determining adrenal dysfunction.

Group III, claim(s) 25-30, drawn to a method for identifying a compound that affects a steroid pathway.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The three groups do not have a Unity of Invention because they are not an accepted combination of inventions (see MPEP annex B Part I section (e) pages AI-36 and AI-37) as they are methods used perform different functions (as described above).

Continuation of B. FIELDS SEARCHED Item 3:
MEDLINE, CANCERLIT, BIOSIS, CAPLUS, REG, BIOTECHDS, EMBASE, WPIDS

search terms: compounds registry numbers and names from figure 2, mass spec?, lc/ms, (cushing? or addison?) (w) disease, adren!l (w) d!sfunction, deficiency

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/16028

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : G 01 N 35/00
US CL : 436/43
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 436/43

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	KAO et al. Diagnosis of adrenal cortical dysfunction by liquid chromatography-tandem mass specrometry. Annals of Clinical and Laboratory Science. April 2001, Vol. 31, No. 2, pages 199-204. See entire document.	1-30
X	JOOS et al. Liquid chromatography-tandem mass spectrometry of some anabolic steroids. Anal. Chem. 15 October 1999, Vol. 71, pages 4701-4710, see especially abstract, Table 2, Figure 12 and conclusion.	1-24
X	SHACKLETON, C.H.L. Mass spectrometry in the diagnosis of steroid-related disorders and in hypertension research. J. Steroid Biochem. 1993, Vol. 45, No. 1, pages 127-140, especially Table 1 and 2, Figure 13 and pages 138 and 139.	1-30
X	VOLMER et al. Rapid determination of corticosteroids in urine by cobined solid phase microextraction/liquid chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry. 1997, Vol. 11, pages 1926-1934, especially abstract, Table 1-3, and conclusion.	1-24

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
06 August 2001 (06.08.2001)

Date of mailing of the international search report
31 OCT 2001

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/16028

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GLASS et al. Steroid sulphatase deficiency is the major cause of extremely low oestriol production at mid-pregnancy: A uninary steroid assay for the discrimination of steroid sulphatase deficiency from other causes. Prenatal Diagnosis. August 1998, Vol. 18, pages 789-800, especially abstract, Figures 1 and 5, and pages 794 and 798.	1-30
X	MA et al. Determination of steroids by liquid chromatography/mass spectrometry. J. Am. Soc. Mass Spectrom. September 1997, Vol. 8, pages 1010-1020, especially abstract, Table 1 and Figure 7.	1-24
X	YERGEY et al. Direct determination of human urinary cortisol metabolites by HPLC/CRIMS. Steroids, March 1995, Vol. 60, pages 295-298, especially abstract, Figure 1, Table 1 and page 298.	1-24